EXHIBIT G

Eur. J. Biochem. 121, 249–257 (1982)

Nucleotide Sequence and Secondary Structure of Citrus Exocortis and Chrysanthemum Stunt Viroid

Hans J. GROSS, Guido KRUPP, Horst DOMDEY, Manfred RABA, Peter JANK, Christine LOSSOW, Heidemarie ALBERTY, Karla RAMM, and Heinz L. SÄNGER

Max-Planck-Institut für Biochemie, Martinsried bei München, and Arbeitsgruppe Pflanzenvirologie, Institut für Virologie, Gießen

(Received July 22, 1981)

The complete nucleotide sequence of citrus exocortis viroid (CEV, propagated in Gymura) and chrysanthemum stunt viroid (CSV, propagated in Cineraria) has been established, using labelling *in vitro* and direct RNA sequencing methods and a new screening procedure for the rapid selection of suitable RNA fragments from limited digests.

The covalently closed circular single-stranded viroid RNAs consist of 371 (CEV) and 354 (CSV) nucleotides, respectively. As previously shown for potato spindle tuber viroid (PSTV, 359 nucleotides), CEV and CSV also contain a long polypurine sequence. Maximal base-pairing of the established CEV and CSV sequences results in an extended rod-like secondary structure similar to that previously established for PSTV and as predicted from detailed physicochemical studies of all these viroids. Although the three viroid species sequenced to date differ in size and nucleotide sequence, there is 60–73% homology between them. As PSTV, CEV and CSV also contain conserved complementary sequences which are separated from each other in the native secondary structure. We postulate that the resulting 'secondary' hairpins, being formed and observed *in vitro* during the complex process of thermal denaturation of viroid RNA, must have a vital, although yet unknown, function *in vivo*. The possible origin and function of viroids are discussed on the basis of the characteristic structural features and of a considerable homology with U1a RNA found for a region highly conserved in the three viroids.

Viroids are small infectious RNAs which may cause disease symptoms in higher plants. As far as is known to date, they seem to exist mainly as single-stranded circular molecules with a unique secondary structure due to intramolecular base-pairing. Nine viroids deriving from different host plants have been described (reviewed by Gross and Riesner [1]). Their occurrence in, and isolation from, different sources, however, does not necessarily mean that all of these nine isolates represent different molecular species. So far this has been shown only for some of the viroids on the basis of their different physical properties and the marked differences between their oligonucleotide fingerprints [2, 3]. Potato spindle tuber viroid (PSTV) is the only viroid which has been described in detail [4, 5].

Unusual properties of PSTV are: (a) a single-stranded RNA with a base composition which nearly fulfills the Chargaff rules for double-stranded DNA [6]; (b) an extended rod-like secondary structure characterized by a sequential arrangement of short base-paired regions and internal loops; (c) the relative frequency of G · U base pairs [4]; (d) the formation of new hairpin structures and consequently the coordinated formation of branched intermediates during thermal denaturation [5].

Abbreviations. CEV, citrus exocortis viroid; CSV, chrysanthemum stunt viroid; PSTV, potato spindle tuber viroid.

Enzymes. RNase A (EC 3.1.27.5); RNase T_1 (EC 3.1.27.3); RNase U_2 (EC 3.1.27.4); polynucleotide kinase (EC 2.7.1.78); DNA-dependent RNA polymerase (EC 2.7.7.6); *Naja oxiana* nuclease (EC 3.1.–.–); alkaline phosphatase (EC 3.1.3.1); *Staphylococcus* nuclease (EC 3.1.31.1); *Neurospora* nuclease (EC 3.1.30.1); nuclease P_1 (EC 3.1.30.1).

There is evidence that all viroids studied until now have similar properties, e.g. molecular size, secondary structure and the complex mechanism of thermal denaturation [5, 7, 8]. On the other hand, it has been demonstrated that viroid isolates of different origin have different fingerprint patterns [3]. Strains of PSTV differing in pathogenicity have been differentiated by their oligonucleotide fingerprints [9]. Finally it has been shown by complete sequence analysis that a pathogenic and a virtually non-pathogenic PSTV isolate differ by three nucleotide exchanges only [10].

In this paper we report the complete primary and secondary structures of CEV and CSV, and compare these with the previously established structure of PSTV. On the basis of these results, we discuss general features of viroid structure, function and origin.

RESULTS AND DISCUSSION

Sequence Analysis

The sequence determination of a linear RNA is highly facilitated by the possibility of labelling its 5′ and 3′ ends *in vitro*. The consequent application of new rapid methods thus establishes the primary structure by proceeding from either end far into the molecule. It is a major handicap that this approach cannot be applied to viroids. Due to their circularity, these RNAs have to be degraded by limited digestion before end-labelling. Again due to their circular structure, very complex mixtures of often more than a 100 overlapping, long fragments are generated and have to be terminally labelled for two-dimensional separation on poly-

acrylamide gels and sequence analysis. As a consequence of the pronounced viroid secondary structure, however, preferential cleavages sites are present in the molecule and thus many of these fragments have the same 5′ end and differ at their 3′ end only, or *vice versa*. Therefore, while searching for a few missing overlaps, the regions around these cleavage sites will be obtained preferentially, and unnecessarily sequenced, again and again. A rapid screening method presented here considerably helps to avoid such multiple sequence work. For this purpose aliquots of the labelled fragments are submitted to controlled acid hydrolysis in the presence of 6 M urea, and analyzed by polyacrylamide gel electrophoresis and autoradiography. The position of guanosines can be recognized by an increased distance between bands in the nucleotide ladder of the sequencing gel [11].

The guanosine maps (Fig. 1A) of a random selection of long fragments clearly show that in this case six out of ten fragments have the same 5′ end or derive from only slightly differing cleavage sites. As indicated in Fig. 1B, this simple and rapid guanosine mapping makes it possible to select overlapping fragments covering the whole RNA and to estimate the total number of nucleotides.

The nucleotide sequences of CEV and CSV were established by (a) analysis of complete RNase T_1 and RNase A fingerprints and (b) analysis of long fragments on thin polyacrylamide gels after controlled hydrolysis with acid or specific

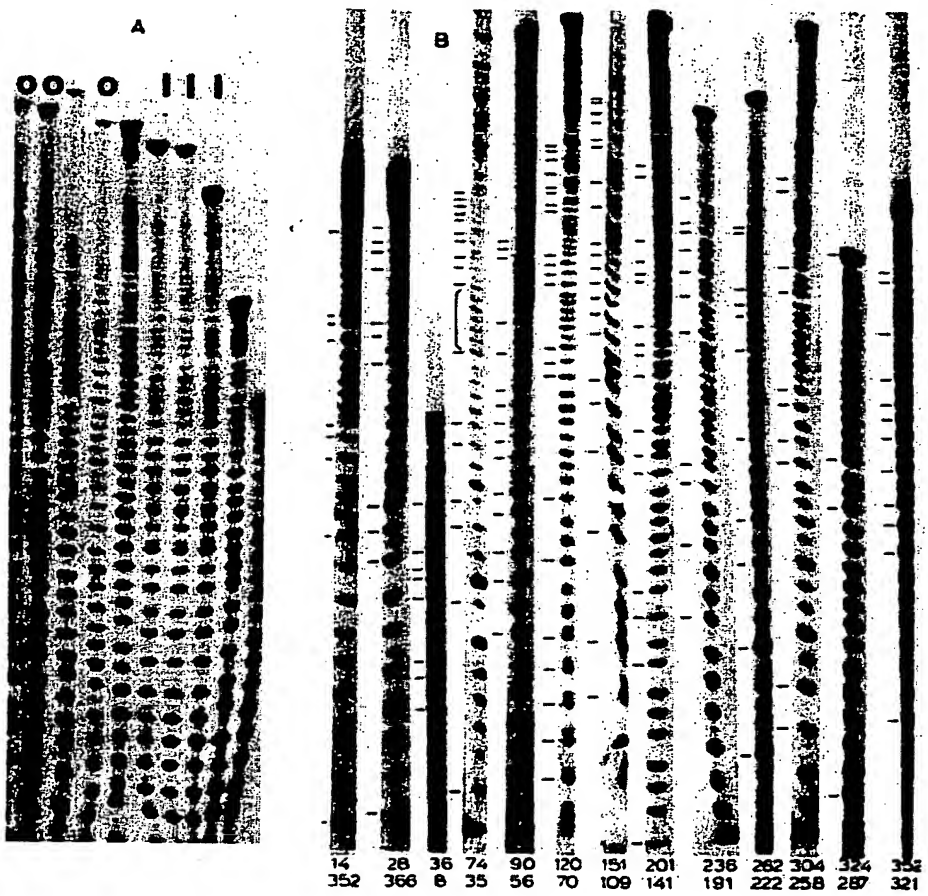

Fig. 1. *Rapid screening of CEV fragments by guanosine mapping.* 5′-^{32}P-labelled CEV fragments, obtained after controlled digestion, were isolated after two-dimensional polyacrylamide gel electrophoresis. An aliquot of each fragment was subjected to controlled acid hydrolysis and separated on 20% polyacrylamide gels. Guanosines can be recognized in corresponding autoradiographs by the characteristic spacing they produce in the *ladders*, i.e. removal of a G results in a more pronounced increase of electrophoretic mobility as compared to A, U or C. Any other random digest, e.g. with alkali, formamide, etc., produces similar results. (A) A random selection of CSV fragments after controlled acid hydrolysis. As can be seen from the guanosine patterns, three fragments derive from identical (I) and three others (O) from only slightly differing cleavage sites. (B) The guanosine mapping of 13 long CEV fragments which had been obtained by a variety of controlled enzymatic digestions. They were combined after the CEV sequence had been completed in order to present a collection of fragments comprising the whole viroid. Guanosines are marked by lines at the left of the ladders. Numbers below each ladder specify the first (bottom) and the last (top) identified guanosine. Numbering of nucleotides is as in Fig. 4. The limitations of this rapid screening method become evident here: not all of the guanosines marked by lines are readily recognized from the gel pattern alone, due to compression of bands in regions of stable secondary structure [5]. The major value of this guanosine mapping is that it helps to identify rapidly the large number of more-or-less identical fragments present in controlled digests. The fragment in lane four derives from a minor CEV species (comprising less than 10% of the CEV isolate) which contains five instead of six adenosines (nucleotides 56–61). A bracket indicates the corresponding nucleotides

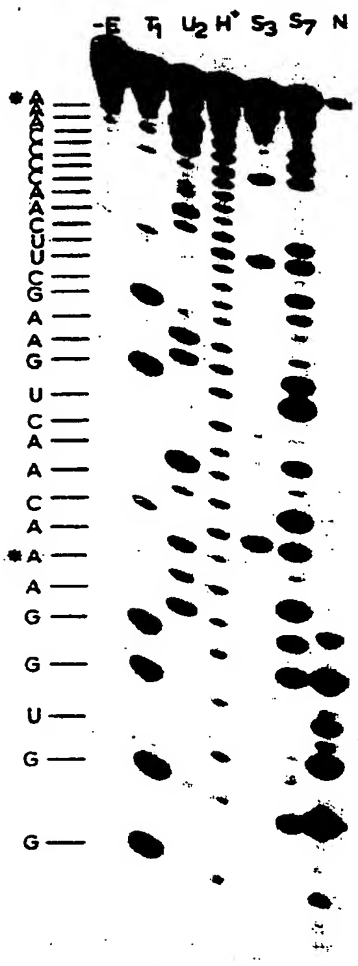

Fig. 2. *Sequence of a long 5'-labelled CEV fragment.* The CEV fragment was partially digested with several base-specific nucleases in the presence of 8 M urea and analyzed on a 20% thin (0.35-mm) gel of which an autoradiograph is presented. Lanes: −E, no enzyme added; T_1, U_2, digestion with corresponding RNases; H^+, controlled acid hydrolysis at pH 2; S3 (S7) incubation with *Staphylococcus* nuclease at pH 3.5 (pH 7.5 with 10 mM Ca^{2+}); N, incubation with *Neurospora* nuclease [41]. An RNase T_1 fingerprint analysis together with sequence determination of the resulting fragments by limited nuclease P_1 digest and mobility shift analysis showed that A-U-A-C-A-A-C-U-G and A-A-A-C-A-A-C-U-G or C-U-U-C-A-A-C-C-C-C-A-A-G and C-U-U-C-A-A-C-C-C-C-A-A-A-C-C-G, respectively, occurred in half molar ratios. RNase A fingerprint analysis demonstrated that A-A-A-C (0.5 mol), A-A-G-C (0.5 mol) and G-G-A-U (2.5 mol) were present in non-integral amounts. From these results two sequence heterogeneities at positions 278 and 301 became obvious (Fig. 4), and thus four different mutants could have been present in our CEV isolate. Sequencing CEV fragments, containing both sites of sequence heterogenity, on polyacrylamide gels demonstrated, however, that only two of these four CEV mutants are present in equimolar ratios: U^{278} is always found together with G^{301}, and A^{278} with A^{301}. A sequence analysis of the latter species is presented here. The corresponding nucleotides are labelled by asterisks

enzymes (Fig. 2), applying 5'-^{32}P-labelling with polynucleotide kinase and [γ-^{32}P]ATP throughout this work. The complete sequences of CEV and CSV are shown in Fig. 3, together with the previously established PSTV primary structure [4].

Although the three viroids differ in the number of nucleotides (Table 1), they contain long stretches of identical sequences. Just as for PSTV, we also have no evidence so far for modified nucleosides in CEV and CSV. Although translation products of viroid RNA have not yet been found [12, 13], it should be mentioned here that CSV, in contrast to CEV and PSTV, contains an AUG sequence (nucleotides 62–64). At least for the circular forms of viroid RNAs, a translation into peptides or proteins seems unlikely since we have recently shown that circularization of a natural ribosome-binding sequence completely abolishes its binding capacity for eukaryotic ribosomes [14].

An interesting observation is the obvious molecular heterogeneity of CEV: adenosines 278 and 301 are replaced partly by uridine 278 and guanosine 301. From RNase T_1 fingerprints we could estimate that the corresponding oligonucleotides occur in nearly equal amounts in our CEV isolate. Moreover, sequence determination of long CEV fragments showed that adenosines 278/301 and uridine 278/guanosine 301 always occur together (Fig. 2) in one sequence. In no case did we find adenosine 278 and guanosine 301 or uridine 278 and adenosine 301 together in an isolated fragment. This may indicate that both CEV entities occur and replicate independently, and that there are no recombinants between the two. Moreover, less than 10% of our CEV isolate contained five instead of six adenosines at the 3' side of guanosine 55. Since even a very few (only three) nucleotide changes may render a severely pathogenic PSTV species virtually non-pathogenic [10], these observations raised the question whether the disease symptoms of the infected host plants are caused by all or only one of these molecular species.

Secondary Structure

Viroids are the only pathogens which do not seem to contain information for peptides or proteins, but code for their own structure only. Consequently, viroid replication and circularization would have to depend completely on host enzymes, and the pathogenic symptoms should result from a direct interaction with certain host components and/or metabolic pathways. This idea was confirmed by our recent finding that viroid RNA binds to, and is transcribed into, complementary RNA of full length, by host plant DNA-dependent RNA polymerase II [15].

The concept of direct viroid-host interactions should be reflected in the presence of, although yet unknown, specific recognition sites for polymerase interaction, initiation, elongation and termination of replication, ligation, pathogenic activities, etc. As shown in Fig. 4, all three viroids can be arranged on the basis of maximum base-pairing into well defined secondary structures of impressive similarity. There is biochemical and physicochemical evidence that these rod-like structures represent isolated viroids in their native state (*in vitro*). Other possible secondary structures with significantly less base pairs and occurring as branched molecules may exist *in vivo* and become stabilized by their intercation with components of the host cell. In fact, metastable structural intermediates were observed after heating and rapid cooling of viroid RNA [5]. We will show below that there is even more similarity between the three viroids than suggested by the overall rod-like secondary structures alone.

Comparison of Three Viroids

The nucleotide composition and the number and type of base pairs of CEV, PSTV and CSV are compared in Table 1.

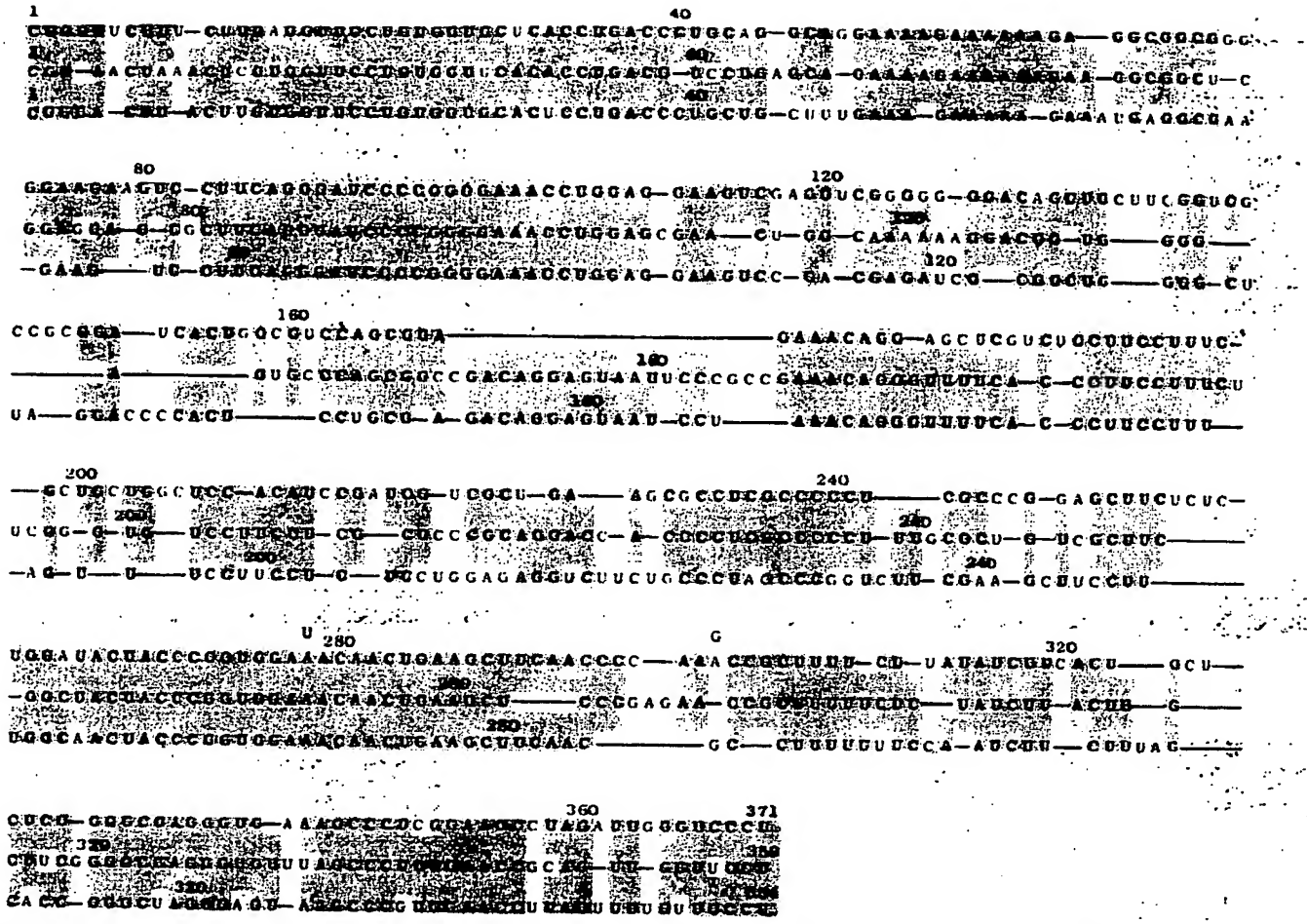

Fig. 3. *Primary structures of CEV, PSTV and CSV (top to bottom).* Sequences are aligned for maximum homology; numbering is as in Fig. 4. Homologous sequences are indicated by shaded areas. A considerable homology between the different viroids is found: CEV-PSTV, 73%, CSV-PSTV, 73%, CEV-CSV, 67%, CEV-CSV-PSTV, 59%

Table 1. *Comparison of nucleotide composition, and number and type of base pairs of CEV, PSTV and CSV*

Viroid	Total number of nucleotides	Number of nucleotides and proportion of total								Ratio		Number of base pairs			
		A		U		C		G		A/U	G/C	total	G·C	A·U	G·U
		no.	(%)	no.	(%)	no.	(%)	no.	(%)						
CEV[a]	371	71 (73)	19.1	77 (76)	20.8	112 (112)	30.2	111 (110)	29.9	0.92 (0.96)	0.99 (0.98)	122 (120)	74 (73)	37 (36)	11
PSTV	359	73	20.3	77	21.4	108	30.1	101	28.1	0.95	0.94	124	72	36	16
CSV	354	74	20.9	93	26.3	97	27.4	90	25.4	0.80	0.93	120	67	41	12

[a] The data for the mutant with adenosines at positions 278 and 301 are given in brackets.

Even these data reveal certain common properties: (a) G:C, A:U and purine:pyrimidine ratios are around one; (b) there are almost twice as much G · C as A · U pairs; (c) 9–13% of the base pairs are G · U pairs, and (d) the overall number of base pairs (120–124) is remarkably constant although the total number of nucleotides differs among the three viroids.

As already mentioned earlier, the viroids contain a long polypurine sequence around nucleotides 50–65. The detailed sequence homology is presented in Fig. 3, where shaded areas identify homologous structures. These homologous sequences, however, are not evenly distributed along the molecules. Rather surprisingly they are accumulated around the first and

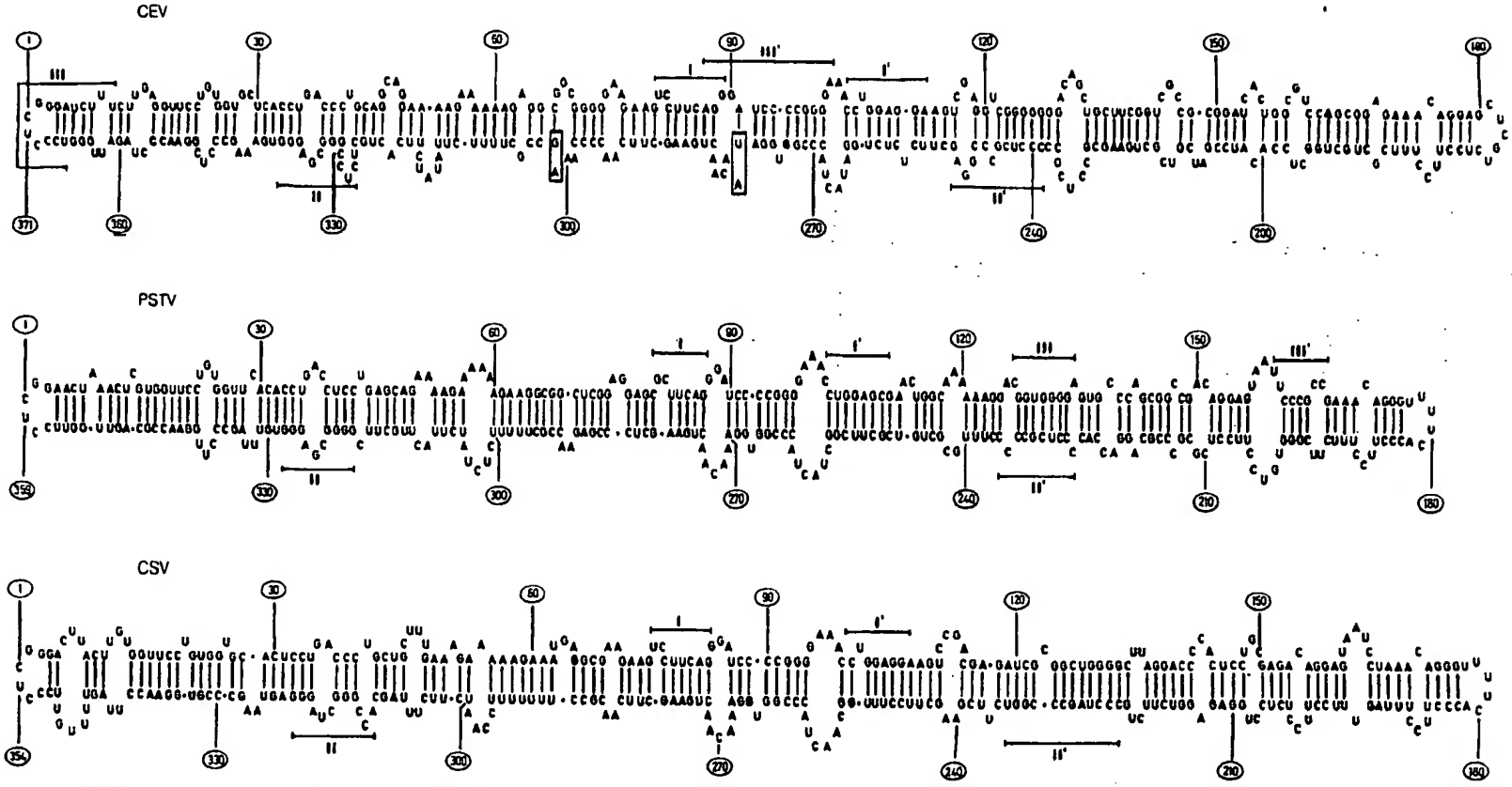

Fig. 4. *Comparison of secondary structures of CEV, PSTV and CSV (from top to bottom).* The sequences are arranged to obtain a maximum number of base pairs and taking in consideration the sensitivity of specific sites to controlled enzymatic digestions. This results in the typical rod-like structure. Arrangements with minor differences are possible for several sections. The first possible A and U or G and C pairs in the end loops are considered as non-hydrogen-bonded on the basis of their reactivity [4,31]. The segments which could form hairpin structures upon thermal denaturation are indicated by lines, indexed I, I'; II, II' and III, III'. Segments I and I' form hairpin I etc. The hairpins are shown in Fig. 5. Evidently hairpins I and III in CEV can not exist simultaneously; the low stability of hairpin III eliminates the possibility of its occurrence

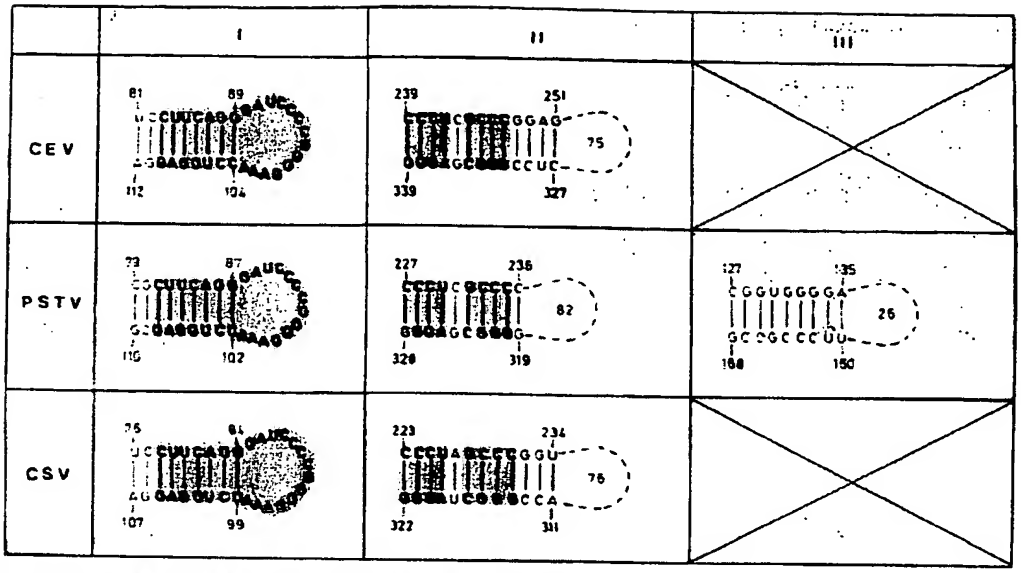

Fig. 5. *Hairpins occurring in intermediate structures during thermal denaturation of viroids.* Sequence identity of hairpin stems (I and II) and loops (I) is indicated by shaded background. Numbers in the loops specify the number of nucleotides in the corresponding loop

the last approximately 110 nucleotides, i.e. there is less homology in the middle of the RNAs. The ends and the central region of the linearized sequences (Fig. 3), however, comprise the left and the right halves, respectively, of the circular viroid secondary structures shown in Fig. 4. Consequently, these viroids in their native state contain two domains: a longer one of significantly more homology on the left as compared to a shorter one, with less homology, to the right. Interestingly, the secondary structure of the left halves appears to be less stable than that of the right halves. The question arises here whether these observed homologies are simply the reflection of a phylogenetic relationship among these viroids, or whether it is the result of a convergent development towards a common functional requirement. An answer to this question is not yet possible. We have, however, to assume that in these small molecules of such sophisticated secondary structure, a great deal of the observed homology has something to do with common functions. This idea is supported by the following considerations concerning the structural transitions of viroids during thermal denaturation. It has been found for PSTV and it had been postulated for all viroids studied to date, that this process is of defined complexity and highly cooperative: melting of the left half of the viroid permits the formation of new ('secondary') and more stable hairpin structures between denatured and yet double-stranded regions. These interactions across the molecule destabilize the corresponding double-stranded domain and lead, in the main transition, to a branched intermediate which, in case of PSTV, contains three 'secondary' hairpins [5,8].

In fact we found complementary sequences with the potential of forming new 'secondary' hairpins in the native structures of CEV and CSV as well. These sequences are marked with bars and Roman numerals in Fig. 4, and are presented as hairpins in Fig. 5. Several features of these structures deserve special interest.

(a) CEV and CSV contain complementary structures for the formation of two 'secondary' hairpins, whereas PSTV forms three such structures during thermal denaturation.

(b) The nucleotides in seven out of nine base pairs and the 14 loop nucleotides are identical in hairpins I (complementary sequences I and I' in Fig. 4) of all three viroids.

(c) In complementary (replicative intermediate) viroid RNAs, hairpins I would be less stable due to an unpaired A and C in the center of the stem deriving from the G · U pair in the stem (Fig. 5).

(d) The potential hairpins II (complementary sequences II and II', Fig. 4) differ slightly in the three viroids, both as far as nucleotide composition and number of base pairs and nucleotides in stem and loop, respectively, are concerned. The long stem region is especially G + C-rich and would be the same in a complementary viroid RNA.

(e) CEV has two partially complementary sequences III and III'. A hairpin formation, however, seems unlikely since this would interfere with the formation of the more stable hairpin I (see Fig. 4 and legend).

(f) The sequences for the 'secondary' hairpins I and II are not only almost identical (I) or very similar (II), but, most interestingly, they are located exactly in the same region of the primary and secondary structure of all three viroids.

These observations together indicate that the 'secondary' hairpins should have a vital function for the viroids *in vivo*. It has been proposed that these hairpins could form primarily during viroid replication, helping to bring the ends together for ligation, i.e. ring formation [16]. A similar mechanism has been proposed for the circularization of the single-stranded DNA of bacteriophage fd [17]. The constant regions, including the characteristic polypurine sequence, however, could also be signals for initiation, elongation or termination of viroid replication. The exact knowledge of more viroid primary and secondary structures will finally enable us to understand and to explain, on the molecular level, the recent finding that viroids directly interact with host RNA polymerase II *in vitro* [15] and *in vivo* [18] and are transcribed by this enzyme into complementary RNA.

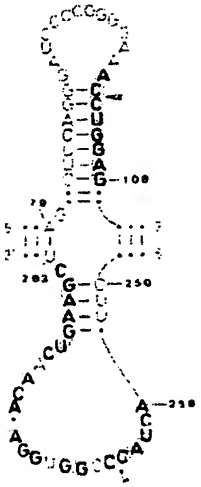

Fig. 6. *Possible palindromic arrangements of a highly conserved region in the center of viroid secondary structure.* Black dots indicate different nucleotides in homologous positions, whereas a line in the nucleotide sequence indicates major differences. Nucleotides identical in PSTV, CEV and CSV are presented in a palindrome-like structure. Nucleotide numbers refer to PSTV (Fig. 3 and 4). Shaded nucleotides are homologous with the 5′ end of U1a RNA from chicken [26], shown in detail for nucleotides 258–282 only, in Fig. 7. The stem region of the hairpin pointing downwards is longer and more stable for the three individual viroids than that shown above for the nucleotides identical in the three viroids

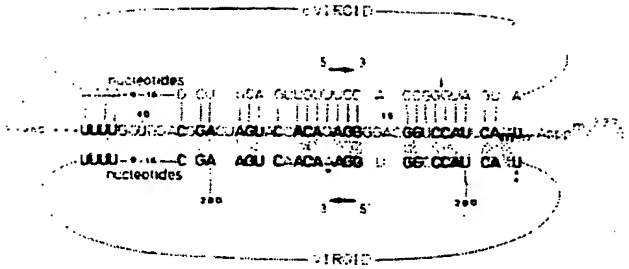

Fig. 7. *Homology of a highly conserved viroid sequence with, and complementarity of the corresponding complementary viroid (cVIROID) to, the 5′-terminal sequence of U1a RNA from chicken.* Nucleotide numbers in the viroid sequence (below) refer to PSTV (Fig. 3 and 4). Homology and complementarity to U1a RNA are presented in detail for nucleotides 258–282 from Fig. 6 only. For a possible significance of the uridine stretch (nucleotides 43–46) of U1a RNA see Results and Discussion. Shaded nucleotides indicate homology between the three viroids and the 5′ end of U1a RNA. Possible base pairs between a complementary viroid and the 5′ end of U1a RNA are indicated by vertical bars. The vertical arrow points to a hypothetical splice junction. A* indicates that a CEV mutant contains U in this position; U** indicates that CSV contains A in this position

Possible Relations between Viroids, Introns and U1a RNA

Nothing is known about the phylogenetic origin of viroids, and it has been speculated that they could be primitive or degenerated viruses [19], or that they might derive from normal cellular RNAs of their hosts [20]. In fact, the possibility has been discussed that they might have originated from circularized introns which have escaped degradation and became self-reproductive [20–22]. Intron-derived circular RNA has been found in yeast mitochondria [23] and a circularized intron sequence even of viroid size has been detected recently in nuclei of *Tetrahymena thermophila* [24].

With the idea in mind that viroids could be 'escaped' introns, we analyzed our viroid sequences for possible relicts of their hypothetical origin. In addition, the detection of several viroid complementary RNA species in viroid-infected plants [18] prompted us to consider also this type of complementary viroid RNA as the 'escaped' intron, from which the infectious circular viroid RNA, accumulating in the infected plant, could be transcribed.

A most interesting result of our analysis was that, in three viroids, highly conserved regions were found, and that there is a surprising sequence homology between U1a RNA and one of these constant regions in the center of the viroid secondary structure (Fig. 6 and 7). U1a RNA, a small nuclear RNA [25], has been highly conserved during evolution [26] and is thought to be involved in eukaryotic mRNA processing by correctly aligning the pre-mRNA intron-exon junctions to be spliced [27, 28].

The A-C-C-U-G sequence of U1a RNA which might be involved in the recognition of pre-mRNA splice junctions and similar sequences (e.g. A-C-C-C-G) occur in several locations of the three viroids. One of these homologous regions (nucleotides 101–108 in PSTV) is part of the stem region of the 'secondary' hairpin I (Fig. 5) of a metastable viroid conformation. A more extended homology, however, is found from nucleotides 258 to 282 (PSTV) in the opposite region of the viroid secondary structure (Fig. 4). This latter region, homologous to the 5′ end of U1a RNA, is part of a very stable double-stranded segment of the three viroid secondary structures *in vitro*. Secondary structure *in vivo*, however, may be different from that shown in Fig. 4. If interaction with cellular components of the host plant favored and stabilized the highly conserved 'secondary' hairpin I, the opposite region in Fig. 4 could also form a hairpin, thus giving rise to a palindrome-like structure (Fig. 6). In this case, the extended sequence homologous to U1a RNA (nucleotides 258–282 in PSTV) becomes mainly single-stranded (Fig. 6). Both stretches of shaded sequences in the palindrome (Fig. 6) are homologous to the region of U1a RNA believed to be directly involved in RNA splicing.

A viroid of complementary sequence (a replicative intermediate) would also be able to form a corresponding palindrome-like structure which would be able to form base pairs with U1a RNA. The arrows in Fig. 6 point to possible splice sites if complementary viroids had originated from circularized introns, according to the model of Lerner et al. [27] and Rogers and Wall [28]. Fig. 7 summarizes (a) the extensive sequence homology between the 5′ end of U1a RNA and a conserved region of the three viroids, and (b) the extensive base-pairing possible between U1a RNA and the corresponding sequence of a complementary viroid. The vertical arrow points to a hypothetical splice site in the complementary viroid. Moreover, viroids contain a conserved oligo(uridine) sequence which corresponds to an oligo(uridine) stretch in U1a RNA. Consequently, complementary viroids contain an oligo(adenosine) sequence, also occurring frequently in introns, 50–80 nucleotides upstream of the splice site of eukaryotic pre-mRNA. The U1a RNA from plants has not yet been characterized; however, intervening sequences with splice junctions similar to those of animal origin have been detected [29].

All these considerations reveal the possibility of relations between introns, U1a RNA structure and/or function, and viroids. In this context it has to be kept in mind that viroids

and complementary viroids, due to their partial self-complementarity, both contain sequences homologous with the 5′ end of U1a RNA, and corresponding nucleotide stretches complementary to the 5′ end of U1a RNA.

Considering the pathogenic activity of viroids, it appears possible that they could interfere with splicing processes both due to their sequence homology with and their base complementarity to the U1a RNA sequence involved in the correct recognition of intron-exon junctions.

Although these ideas do not provide any definite conclusions about the origin of viroids, it is still an atractive working hypothesis that they, or perhaps more likely their complementary counterparts, may have originated from circularized introns which were stabilized due to their high degree of self-complementarity and acquired the ability of self-replication.

Note. When this manuscript was prepared for submission, the nucleotide sequence and secondary structure of a different CSV isolate was published by Haseloff and Symons [30]. Their CSV strain differs in 10 nucleotides and in the total nucleotide number from our isolate.

EXPERIMENTAL PROCEDURES

Propagation and Purification of Viroids

CEV and CSV were propagated in and isolated from *Gynura auranthiaca* and *Cineraria hybrida*, respectively. Purification of viroids was done as described [2].

Fingerprint Analysis

Complete digestions of viroid RNAs with RNase T_1 and RNase A, 5′-^{32}P-labelling, fingerprint analyses and sequence determination were performed as described [31].

Controlled Nuclease Digestions of Viroids

Digestions were done essentially as described [4]. Additionally several different nucleases and modified conditions have been applied.

(a) 5 µg viroid RNA were incubated with 0.03 U RNase T_1 or 0.3 U RNase U_2 in 2 µl 15 mM Tris/HCl (pH 7.4), 175 mM KCl, 10 mM $MgCl_2$ for 1 h at 37 °C.

(b) 2 µg viroid RNA were digested with 0.8 µg *Naja oxiana* nuclease [32] in 5 µl 25 mM Tris/HCl (pH 7.4), 15 mM $MgCl_2$, 280 mM KCl, 5 mM mercaptoethanol for 30 min in ice. The nuclease was inactivated by adding 2 µl diethylpyrocarbonate. The RNA fragments were recovered by ethanol precipitation and incubated with 0.02 U RNase-free alkaline phosphatase from calf intestine [33] in 10 µl 100 mM Tris/HCl (pH 8.0) for 1 h at 50 °C to remove the 5′-phosphate groups. The phosphatase was inactivated by the addition of 2 µl 50 mM nitrilotriacetic acid, pH 7.2 [34].

(c) 5 µg viroid RNA were incubated in 20 µl 50 mM sodium acetate (pH 4.5) for 2 h at 37 °C with 10 µg acid phosphatase from human prostate [35] which contains an unidentified ribonuclease.

5′-^{32}P-Labelling, Purification and Isolation of Long Viroid Fragments

5′-Labelling *in vitro* with [γ-^{32}P]ATP and T_4 polynucleotide kinase of the fragments was done as described [34]. The labelled fragments were separated by two-dimensional polyacrylamide gel electrophoresis [36] using thin (0.35-mm) gels [37] and eluted from the excised gel pieces essentially as described by Kuchino et al. [38]. The gel pieces were frozen at −20 °C in 300 µl of 0.5 M ammonium acetate, 1 mM $MgCl_2$, 0.1% sodium dodecyl sulfate, 0.1 mM Na_2EDTA, pH 7.0 in the presence of 50 µg yeast tRNA. After thawing they were shaken at room temperature for 8–12 h. The extracted RNA was recovered by ethanol precipitation.

Random Hydrolysis, Rapid Guanosine Mapping and Sequence Analysis of End-Labelled RNA

Hydrolyses were performed in the presence of 6 M urea and 5 µg RNA with 165 mM H_2SO_4 in 5 µl for 3–5 min at 100 °C or as an alternative with 50 mM NaOH for 3–5 min at 80 °C. The samples were separated by electrophoresis on thin polyacrylamide gels [37] and autoradiographed at −70 °C using preflashed X-ray films and intensifying screens [39].

RNA sequence analysis was performed essentially as described [4, 40, 41].

We thank Prof. V. Erdmann for *Naja oxiana* nuclease. This work was supported by *Deutsche Forschungsgemeinschaft* through personal grants (Gr. 576/4-4) and *Sonderforschungsbereich* 47.

REFERENCES

1. Gross, H. J. & Riesner, D. (1980) *Angew. Chem. Int. Ed. Engl. 19*, 231–243.
2. Sänger, H. L., Klotz, G., Riesner, D., Gross, H. J. & Kleinschmidt, A. K. (1976) *Proc. Natl Acad. Sci. USA, 73*, 3852–3856.
3. Gross, H. J., Domdey, H. & Sänger, H. L. (1977) *Nucleic Acids Res. 4*, 2021–2028.
4. Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. & Sänger, H. L. (1978) *Nature (Lond.) 273*, 203–208.
5. Riesner, D., Henco, K., Rokohl, U., Klotz, G., Kleinschmidt, A. K., Domdey, H., Jank, P., Gross, H. J. & Sänger, H. L. (1979) *J. Mol. Biol. 133*, 85–115.
6. Chargaff, E. (1950) *Experientia (Basel) 6*, 201–209.
7. Langowski, J., Henco, K., Riesner, D. & Sänger, H. L. (1978) *Nucleic Acids Res. 5*, 1589–1610.
8. Henco, K., Sänger, H. L. & Riesner, D. (1979) *Nucleic Acids Res. 6*, 3041–3059.
9. Dickson, E., Robertson, H. D., Niblett, C. L., Horst, R. K. & Zaitlin, M. (1979) *Nature (Lond.) 277*, 60–62.
10. Gross, H. J., Liebl, U., Alberty, H., Krupp, G., Domdey, H., Ramm, K. & Sänger, H. L. (1981) *Biosci. Rep. 1*, 235–241.
11. Olins, P. O. & Jones, D. S. (1980) *Nucleic Acids Res. 8*, 715–729.
12. Davies, J. W., Kaesberg, P. & Diener, T. O. (1974) *Virology, 61*, 281–286.
13. Semancik, J. S., Conejero, V. & Gerhart, J. (1977) *Virology, 80*, 218–221.
14. Konarska, M., Filipowicz, W., Domdey, H. & Gross, H. J. (1981) *Eur. J. Biochem. 114*, 221–227.
15. Rackwitz, H.-R., Rohde, W. & Sänger, H. L. (1981) *Nature (Lond.) 291*, 297–301.
16. Gross, H. J. (1980) *Hoppe-Seyler's Z. Physiol. Chem. 361*, 477–492.
17. Schaller, H., Voss, H. & Gucker, S. (1969) *J. Mol. Biol. 44*, 445–458.
18. Rohde, W. & Sänger, H. L. (1981) *Biosci. Rep. 1*, 327–336.
19. Diener, T. O. (1974) in *Viruses, Evolution and Cancer* (Kurstak, E. & Maramorosch, K., eds) pp. 757–783, Academic Press, New York.
20. Diener, T. O. (1979) *Viroids and Viroid Diseases*, John Wiley and Sons, New York, Chichester, Brisbane, Toronto.
21. Roberts, R. J. (1978) *Nature (Lond.) 274*, 530.
22. Crick, F. (1979) *Science (Wash. DC) 204*, 264–271.
23. Halbreich, A., Pajot, P., Foucher, M., Grandchamp, C. & Slonimski, P. (1980) *Cell, 19*, 321–329.

24. Grabowski, P. J., Zaug, A. J. & Cech, T. R. (1981) *Cell, 23*, 467–476.
25. Reddy, R., Ro-Choi, T. S., Henning, D. & Busch, H. (1974) *J. Biol. Chem. 249*, 6486–6494.
26. Branlant, C., Krol, V., Ebel, J. P., Lazar, E., Gallinaro, H., Jacob, M., Sri-Widada, J. & Jeanteur, P. (1980) *Nucleic Acids Res. 8*, 4143–4154.
27. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. & Steitz, J. A. (1980) *Nature (Lond.) 283*, 220–224.
28. Rogers, J. & Wall, R. (1980) *Proc. Natl Acad. Sci. USA, 77*, 1877–1879.
29. Sun, S. M., Slightom, J. L. & Hall, T. C. (1981) *Nature (Lond.) 289*, 37–41.
30. Haseloff, J. & Symons, R. H. (1981) *Nucleic Acids Res. 12*, 2741–2752.
31. Domdey, H., Jank, P., Sänger, H. L. & Gross, H. J. (1978) *Nucleic Acids Res. 5*, 1221–1236.
32. Vasilenko, S. & Babkina, V. (1965) *Biokhimiya, 30*, 705–712.
33. Efstratiadis, A., Vournakis, J. N., Donis-Keller, H., Chaconas, G., Dougall, D. K. & Kafatos, F. C. (1977) *Nucleic Acids Res. 4*, 4165–4174.
34. Silberklang, M., Gillum, A. M. & RajBhandary, U. L. (1979) *Methods Enzymol. 59*, 58–109.
35. van Etten, R. L. & Saini, M. S. (1978) *Clin. Chem. 24*, 1525–1530.
36. Lockard, R. E., Alzner-De Weerd, B., Heckman, J. E., MacGee, J., Tabor, M. W. & RajBhandary, U. L. (1978) *Nucleic Acids Res. 5*, 37–56.
37. Sanger, F. & Coulson, A. R. (1978) *FEBS Lett. 87*, 107–110.
38. Kuchino, Y., Kato, M., Sugisaki, H. & Nishimura, S. (1979) *Nucleic Acids Res. 6*, 3459–3469.
39. Laskey, R. A. & Mills, A. D. (1977) *FEBS Lett. 82*, 314–316.
40. Donis-Keller, H., Maxam, A. M. & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2537.
41. Krupp, G. & Gross, H. J. (1979) *Nucleic Acids Res. 6*, 3481–3490.

H. Gross and G. Krupp, Institut für Biochemie der Julius-Maximilians-Universität Würzburg, Röntgenring 11, D-8700 Würzburg, Federal Republic of Germany

H. Domdey, Institut Suisse de Recherches Experimentales sur le Cancer, Chemin des Boveresses, CH-1066 Epalinges, Vaud, Switzerland

M. Raba, P. Jank, C. Lossow, H. Alberty and H. Sänger, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-8033 Martinsried, Federal Republic of Germany

K. Ramm, Arbeitsgruppe Pflanzenvirologie der Justus-Liebig-Universität, Schubertstraße 1, D-6300 Gießen, Federal Republic of Germany